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Scientific Officer
Patrick Curran, CAPT, MC, USN
Naval Medical R&D Command
Director of Research and Development
Bethesda, MD 20814-50440

Ref: N00014-91-C-0044

Dear Captain Curran:

Enclosed is the Fourth Triannual Report for Contract No.: N00014-91-C-0044, which is entitled "Cellular and Tissue Injury During Nonfreezing Cold Injury and Frostbite". This Report covers the period from January - April, 1992. If you have any questions about the Report or the research, please contact me at 404-952-1660.

Sincerely yours,

John F. Carpenter, Ph.D.
Senior Scientist

cc: Mrs. Mellars, DCMDS-GAACA
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Cellular and Tissue Injury During Nonfreezing Cold Injury and Frostbite

Fourth Triannual Report: January - April, 1992

Metabolic perturbation studies. We have completed a detailed statistical analysis of our metabolic heat rate and cross-over point data for red blood cells. Both data sets support the original hypothesis that the degree of protonation, not pH per se, determines the relative activity of the enzymes in a metabolic pathway. This work and related studies with microcalorimetry will be presented at the 1992 International Meeting of the Society for Cryobiology (Abstracts enclosed).

Analysis of the data from microcalorimetric experiments involved comparison of Q_{10} (i.e., change in rate due to a 10°C change in temperature) values for metabolic heat rates under conditions of constant pH (pH stat) vs. alphastat conditions. Alphastat means that the degree of protonation of alpha imidazole in histidine residues is maintained constant as temperature is varied. (Histidine is the only amino acid in enzymes that has a pK_a in the physiological pH range.) In our red cell model, this is accomplished by allowing intracellular pH to increase, as temperature is lowered, to the same degree that histidine pKa increases. The theory predicts that Q_{10} should be higher for biological rate functions under pH stat than under alpha stat conditions. This is because under pH stat the degree of protonation of histidine residues increases as temperature is lowered. For our data, Q_{10} for metabolic heat (which in red cells is predominantly due to glycolytic flux) under pH stat was larger for all pairs of measurements compared to the alphastat pairs of measurements, over the same temperature range. This trend was statistically significant at the $p < 0.05$ level by the paired student's t test. These data represent the first *in vivo* demonstration of this phenomenon for glycolytic regulation.

Cross-over point analysis involves comparing the ratio of the concentration an enzyme's product to that for the substrate (i.e., the mass action ratio). By determining metabolite levels for cells under different conditions, one can determine the relative activity of key regulatory enzymes. Our work is on phosphofructokinase, which regulates glycolytic flux. Thus, when the ratio of fructose 1,6-bisphosphate to fructose-6-phosphate is decreased under a given set of conditions (e.g., acidotic conditions), relative to a reference state (e.g., alkaline pH) we know that phosphofructokinase is inhibited. At a given temperature, the profile of mass action ration vs. intracellular pH is sigmoidal, with minimum and maximum values noted, respectively, at low and high pHi. The alphastat hypothesis predicts that this profile should shift to higher pH's as temperature is lowered. That is, PFK is inhibited to a greater degree at a given pHi as temperature is lowered, because histidine residues are more highly protonated. Shifts in the pH profile of mass action ratios for PFK were determined in two ways. First, specific points in the profiles, at which the first statistically significant increase in the mass actio ratio was noted, were compared for each temperature. This point represents the threshold pHi at which phosphofructokinase activity increases as conditions become less acidotic. A second criterion for these comparisons was that the mass action ratios at these points for each profile were not significantly different from each other. That

is, under these conditions phosphofructokinase has the same relative activity at all temperatures tested. The intracellular pH's of these points were 7.11, 7.24, and 7.36 for 37, 20, and 4°C, respectively and were statistically different from each other (paired student's t test, $p < 0.05$ or lower). Thus, the pHi profile shifted about 0.25 pH units when temperature was lowered from 37 to 4°C ($p < 0.001$).

The second method of determining the pH shifts with temperature was with the use of a curve fitting program, Flexifit. The program fits data to a smoothing spline, and determines four parameters, which it uses to compare shapes and positions of curves. We used the program to determine the pHi at which the mass action ratio curves for 37, 20 and 4°C were at a level of 50% of the maximum values. Values (mean $\pm$ SE) were 7.136 ± 0.007, 7.236 ± 0.022, and 7.389 ± 0.007 for 37, 20, and 4°C, respectively. These analyses provide the first *in vivo* demonstration that alphastat effects are significant in determining the relative activity of phosphofructokinase.

Thermotropic membrane phase transitions and hypothermic injury to cells. We have been working with two human cell lines that are known to produce heat shock proteins (e.g., hsp70) during sublethal thermal stress. Our long-term goal is to discern if the induction of these proteins prior to hypothermic stress increases resistance of the cells to chilling damage. Interestingly, during the control experiments, we discovered that these two cell lines had remarkably different sensitivities to hypothermia. We used leakage of lactate dehydrogenase as an indicator of cell damage. Based on this assay, with one line (MOLT-4) >90% cells were destroyed within 24 hours at 4°C. In contrast, with the other line (K562) more than 72 hours at 4°C was required to induce similar damage. This serendipitous observation provided us with an opportunity to test our hypothesis that thermotropic membrane phase transitions are primary lesions during chilling injury to cells. Using Fourier transform infrared (FTIR) microspectroscopy, we have found that the average, main transition temperature of the membrane phospholipids from liquid crystalline to gel state was much higher (ca. 10°C) for the MOLT-4 than for the K562 cells. Thus, for these two cell lines increased sensitivity to chilling damage correlates with a higher membrane phase transition temperature. This work will be presented at the 1992 Annual Meeting of the Society for Cryobiology (Abstract enclosed).

Our future work on this system will consist of employing more techniques to assess chilling-induced cellular damage, identifying the connection between membrane phase transitions and this damage, and attempts to increase cellular resistance to hypothermic stress by employing membrane fluidizers. We have established an assay for mitochondrial (Krebs cycle) function, which can be used with the intact cells. In addition, we have recently used microcalorimetry to document that total metabolic heat produced by the cells at 37°C is reduced after long-term exposure to 4°C. The degree of the effect is greatest for the MOLT-4 cells, which correlates with our results for lactate dehydrogenase leakage.

With the microcalorimetric and mitochondrial assays, we have begun to screen membrane fluidizing compounds for their acute effects on cellular function at 37°C. In parallel we are using FTIR microspectroscopy to measure the effect of the compounds on thermotropic membrane phase transition temperatures in the cells. The goal is identify a compound, and a concentration of that compound, that lower the membrane phase transition temperature, without altering normal cellular metabolic function at 37°C. Then we can test the effect of the compound

on cells incubated at 4°C, using the post-hypothermia assays at 37°C. If this is not feasible, we will attempt to wash out the fluidizer prior to the assaying the cells at 37°C.

Our hypothesis for the connection between thermotropic membrane phase transitions and chilling-induced cellular injury is that membrane perturbation leads to a loss of calcium homeostasis, i.e., an increase in intracellular calcium levels. The concomitant activation of calcium-dependent proteases and lipases then leads to destruction of key biomolecules and, ultimately, to cell death. The phase transition could lead to increased intracellular calcium because of: 1) direct leakage of calcium into the cell or from intracellular storage organelles because of membrane defects; and 2) greater inhibition of calcium ATPases than calcium channels due to increased membrane viscosity. We will first investigate the role of calcium in chilling-induced cell death and then test means (e.g., membrane fluidization and/or channel blockers) by which to attentuate this effect.

Chilling-induced platelet aggregation and hypersensitivity. One of the primary causes of tissue damage during cold injury is the failure of vascular flow to be re-established to the affected area after warming. As we noted in our last Triannual Report, this no-reflow phenomenon appears to be due primarily to the activation and aggregation of platelets to form thrombi during cold injury. Our working hypothesis is that cold-induced spontaneous aggregation and hypersensitivity to agonists -- phenomena that have been clearly documented in the literature -- are mediated in platelets by an increase in intracellular calcium levels. The loss of calcium homeostasis, in turn, we think is a result of membrane perturbation (e.g., direct leakage due to thermotropic phase transitions). To date, we have set-up a fluorometric assay using FURA to measure intracellular levels of calcium in platelets. The initial experiments indicate that after exposure to 0-4°C, there is leakage of extracellular calcium into the platelet's cytosol. However, these are very early experiments and we will need to characterize in detail the temperature-dependency of this process, and associated platelet hypersensitivity and spontaneous aggregation, before offering any firm conclusions. We expect this work to be a major focus of the laboratory once our platelet aggregometer arrives in June.

Of course, any progress in understanding chilling damage to platelets would also have utility in designing means to store platelets under refrigeration. We will continue to look for these types of long-term, practical applications as our basic, mechanistic studies progress.

Attenuation of ice crystal damage with antifreeze protein. Another proposed area of research under this grant was to investigate the potential usefulness of antifreeze proteins in ameliorating cellular and tissue damage during frost-bite. In addition to depressing the freezing point of water, antifreeze proteins are extremely efficient at inhibiting ice recrystallization in frozen solutions. Knight and Duman have proposed that this may be an important function of the proteins in freeze-tolerant organisms (*Cryobiology* 23, 256-263, 1986). We have tested this proposal *in vitro* by characterizing the influence of antifreeze protein (AFP) on the recovery of cryopreserved cells, which often can survive cooling and yet subsequently be damaged by ice crystal growth during warming. Similar ice crystal damage is thought to occur during re-warming of frostbitten areas. Hence, rapid warming, which minimizes recrystallization, leads to reduced damage. In our studies, rapid warming was not needed to minimize damage to frozen cells, if the cells were frozen and re-warmed in the presence of

antifreeze protein. Relatively low concentrations (e.g., 5 -150 µg/ml) of winter flounder *(Pseudopleuronectes americanus)* AFP enhanced survival of red blood cells cryopreserved in hydroxyethyl starch solutions, an effect that was most apparent in samples warmed at suboptimal rates. Cryomicroscopy demonstrated that AFP inhibited ice recrystallization in the extracellular regions during the latter stages of the warming cycle. AFP concentrations that enhanced survival of red cells conferred partial inhibition. Relatively high concentrations of AFP (e.g, 1.54 mg/ml) were much more effective at inhibiting extracellular recrystallization. However, extensive growth of ice around the cell, and concomitant cell damage, were noted. The mechanism for this AFP-induced ice growth is unknown. We propose that there is a delicate balance between AFP-induced enhancement of cell preservation and of cell damage, which hinges on the degrees of inhibition of ice recrystallization and of preferential growth of ice around the cells. We conclude that, under appropriate conditions, one of the proposed functions of antifreeze proteins in nature can be emulated, and perhaps have application, in cryopreservation of materials of biomedical interest, such as red blood cells. In addition, antifreeze proteins potentially could be used to attenuate the damage, due to migratory recrystallization of ice, that arises when frostbitten areas are rewarmed. One manuscript describing this research has been submitted for publication and another is in preparation. This research will be presented at the 1992 Annual Meeting of the Society for Cryobiology (Abstract enclosed).

Effect of acidosis and ionic environment on skeletal muscle fibers and sarcoplasmic reticulum. Two papers that are in press and describe some our research in this area are enclosed.